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To test our hypothesis that that the HA binding peptide (HABP/tachyplesin) may be a new anti-neurofibromatosis agent, we utilized phage display approach to define its binding target. The results demonstrated that 1) HABP/tachyplesin the binds to collagen-like domain of C1q, a key component in the complement pathway; 2) HABP/tachyplesin appears to activate the classical complement cascade, since it triggered several down-stream events including the cleavage and deposition of C4 and C3 and the formation of C5b-9; 3) The HABP/tachyplesin binding to C1q and activation of classical complement cascade can be blocked if the tumor cells are treated with hyaluronidase or a large excess of hyaluronan, indicating that hyaluronan or related glycosaminoglycans were involved in this process; 4) Treatment of tumor cells with HABP/tachyplesin and serum results in an increase in membrane permeability as indicated by the ability of FITC-dextran to enter the cytoplasm; 5) The combination of HABP/tachyplesin and human serum can markedly inhibit the proliferation of tumor cells and this effect is attenuate if the serum is heat-inactivated or if hyaluronidase is added. This represents a new anti-tumor mechanism.

We will continue to examine the action mechanism of HABP and to test if the *in vitro* anti-tumor effect of HA binding peptide can be translated *in vivo* against the cell growth of neurofibromatosis.

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INTRODUCTION

The loss of neurofibromin, a tumor suppressor corded by NF1 gene, is the major characteristic of neurofibromatosis, which leads to the accumulation of hyperactive Ras-GTP which turns on uncontrolled mitogenic signals in the nucleus (1-5). Therefore, NF1 can be regarded as a disease resulting from the disruption of the balance between cell proliferation and apoptosis (6, 7). In other words, NF1 cells gain immortality due to their over-proliferation and defective apoptosis.

Based on the molecular mechanism of NF1, several strategies have been developed to control the over-growth of NF1 cells, including the induction of cell death via interference with integrity of cell membrane, cell cycle and program death machinery.

We are focused on a unique molecule, hyaluronan (HA) binding peptide (HABP). It is a natural peptide, also called tachyplesin (30), which possesses HA binding activity. It is produced by horseshoe crab. The rational for study of this HABP is based upon the following facts. **1)** Proteins that can bind to HA such as the soluble forms of CD44 and RHAMM can inhibit tumor growth and/or metastasis (8-10). **2)** Fragments of proteins that contain HA binding domain, such as endostatin (fragment of collagen XVIII), angiostatin (fragment of plasminogen), and hemopexin-like domain of metalloproteinase also possess potent anti-tumor activity (11-14). **3)** For more than a decade, the powder or extracts from shark cartilage have been widely used as alternative medicine by cancer patients in USA, Europe and Asia. In some patients, this substance did exhibit anti-tumor effects. Cartilage contains large amounts of HA binding proteins (HABP). It is possible that the anti-tumor effect of shark cartilage achieved in some patients is due to a small amount of HABP passing through impaired intestinal mucosa of these individuals (15-22). And **4)** several proteins purified from the cartilage, a HA-rich tissue, have been found to have the anti-tumor effect (23-29).

In the past, we have demonstrated that HABP exerts an anti-tumor effect on NF1 cells.

In this year, we continue to study the anti-tumor mechanism of HABP by using a phage display approach to define the molecule that interacts with HABP. To our amazement, we found that the C1q, a key component in the complement pathway bound to HABP and activate the classical complement cascade, leading to cell death.

The results are summarized as follows.

BODY

In previous studies, we have demonstrated that the **hyaluronan binding peptide (HABP)** exerts anti-tumor effect on NF1 cells. This HABP is a small peptide composed of 17 amino acids that was initially isolated from the horseshoe crab, also called **tachyplesin** (31). It has an amphipathic structure conferred by two anti-parallel β-sheets held rigidly in place by two disulfide bonds. This structure appears to be critical for its anti-tumor activity (32). However, the initial target of this molecule is largely unknown.

To elucidate the mechanism by which HABP/tachyplesin (briefly tachyplesin) can inhibit the growth of tumor cells, we began by investigating proteins that can bind to tachyplesin. Using the T7 phage display technique, we identified the C1q sub-component of human complement 1 as a potential target. The interaction between C1q and tachyplesin was confirmed by an ELISA and by affinity-precipitation. These results suggest that the binding of tachyplesin to C1q triggers the activation of classical complement pathway and leads to the killing of tumor cells. Interestingly, this effect was blocked if the target cells were pretreated with hyaluronidase or an excess of hyaluronan. This indicates that tachyplesin may initially target hyaluronan and related compounds on the cell surface and subsequently bind to C1q in the serum to activate the complement pathway. This represents a new mechanism for an anti-tumor agent.

1. Isolation and Characterization of Tachyplesin-binding Phages: To identify sequences that bind to tachyplesin, we screened a phage-displayed library of some 1.6×10^7 unique clones expressing sequences from tumor cells ranging in size from 300 to 3,000 base pairs in length fused to the T7 gene 10 capsid protein. Phage particles expressing tachyplesin-binding proteins/peptides were affinity purified on the wells of a microtiter plate coated with tachyplesin or control peptide. After 4 or 5 rounds of bio-panning, the number of phages from the tachyplesin-coated plates was approximately 100-fold greater than that from control peptide plates. Ten plaques were then selected and amplified by PCR. Eight had the same size PCR products and some of these were then sequenced. The deduced amino acid sequences were then subjected to a blast analysis and the results repeatedly identified human C1q, a subcomponent of complement. The region of C1q involved in the binding to tachyplesin was located around the first 400 base pairs of complement C1q B chain open reading frame (Genebank access number NM_000491), corresponding to the N-terminal collagen-like domain of C1q (33).

2. Binding of the C1q to Tachyplesin: To further test the possibility that tachyplesin binds to C1q, we examined the interaction between these two proteins using an ELISA-like system. In the first assay, plates were coated with tachyplesin or the control peptide, probed with C1q and then the amount of bound C1q was detected with anti-C1q.

As shown in **Fig. 1 A,** C1q binds to immobilized tachyplesin in a dose-dependent manner, but not to the control peptide. Similar results were obtained when the plates were pre-coated with C1q and then probed with biotinylated tachyplesin which was detected by a streptavidin coupled system (**Fig. 1 B**). However, this interaction was significantly reduced if the tachyplesin was denatured by reduction and alkylation of the disulfide bonds and further acetylation of the charged side chains (**Fig. 1 C**), which suggests that the interaction between tachyplesin and C1q depends upon the secondary structure of tachyplesin.

The binding of tachyplesin to C1q was also dependent on the NaCl concentration used in the assay buffer (**Fig. 1 D**). The maximum binding occurred at 0.15 M NaCl, the normal physiological salt concentration. Both increasing and decreasing the ionic strength in the assay drastically reduced the binding.

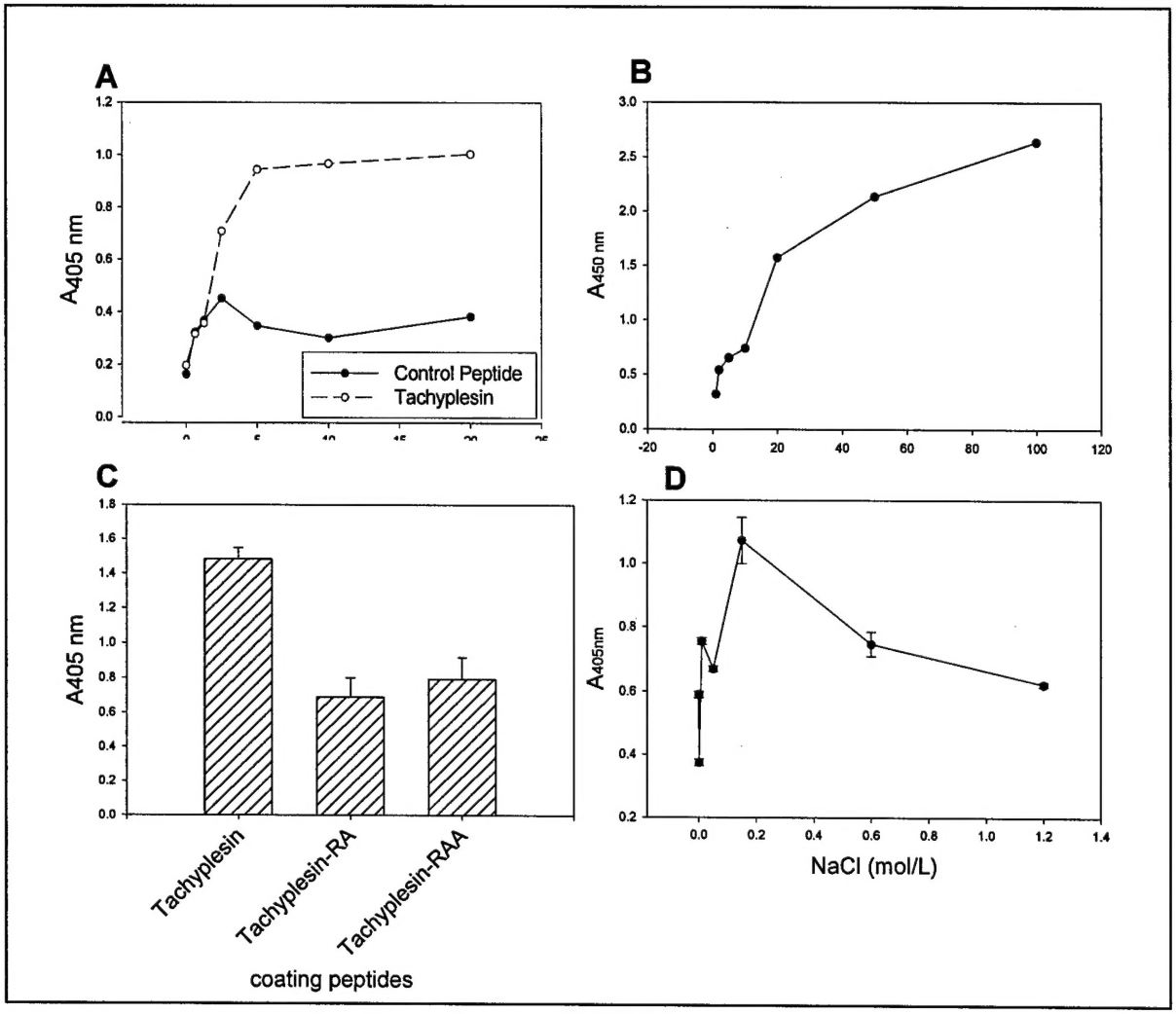

Fig. 1. The interaction between tachyplesin and the C1q subcomponent of complement 1. A) ELISA plates were coated with varying concentrations of either tachyplesin or the control peptide and then incubated with 1 μg of C1q that was then detected with anti-C1q and peroxidase conjugated detection system. The C1q bound to the plate coated with tachyplesin but not to that coated with the control peptide. **B)** Plates were coated with varying concentrations of purified C1q, probed with biotinylated tachyplesin (1 μg/ml) and then assessed with the streptavidin conjugated peroxidase and substrate system. Again, the tachyplesin bound to the immobilized C1q. **C)** Plates were coated with similar amounts (10 μg/ml) of native, reduced and alkylated (RA), and reduced, alkylated and acetylated (RAA) tachyplesin. Then, the plates were probed with 100 ng/ml of C1q followed by anti-C1q and peroxidase conjugated detection system. The binding of C1q to native tachyplesin was significantly greater than either of the denatured peptides. **D)** To test the effect of ionic strength on the interaction, ELISA plate coated with 10 μg/ml of C1q and then incubated with 0.1 μg/ml of biotinylated tachyplesin in VBS buffer of variable ionic strength. The maximum binding corresponded to physiological ionic strength of 0.15 M NaCl.

The interaction between tachyplesin and C1q was further examined by affinity-precipitation and Western blotting of normal human serum. For this, a biotinylated version of tachyplesin was incubated with normal human serum, followed by streptavidin-Sepharose. The immobilized proteins were then eluted and analyzed by Western blotting with anti-C1q polyclonal antibody. As shown in **Fig. 2**, probing the blot with

a poly-clonal antibody revealed three bands corresponding to three chains of the C1q complex (A 27.5, B 25.2, and C 23.8 kDa, respectively) in the tachyplesin treated sample, but not in the samples without the peptide or with the control peptide. Not surprisingly, only a small portion of C1q from the serum was pulled down since serum contains a relatively high concentration of this protein (80 μg/ml) (21). Taken together, these results suggests that C1q binds to both immobilized (surface-bound) and free (liquid-phase) tachyplesin, and tachyplesin binds to both purified and serum C1q, which confirms that there is true interaction between these two molecules.

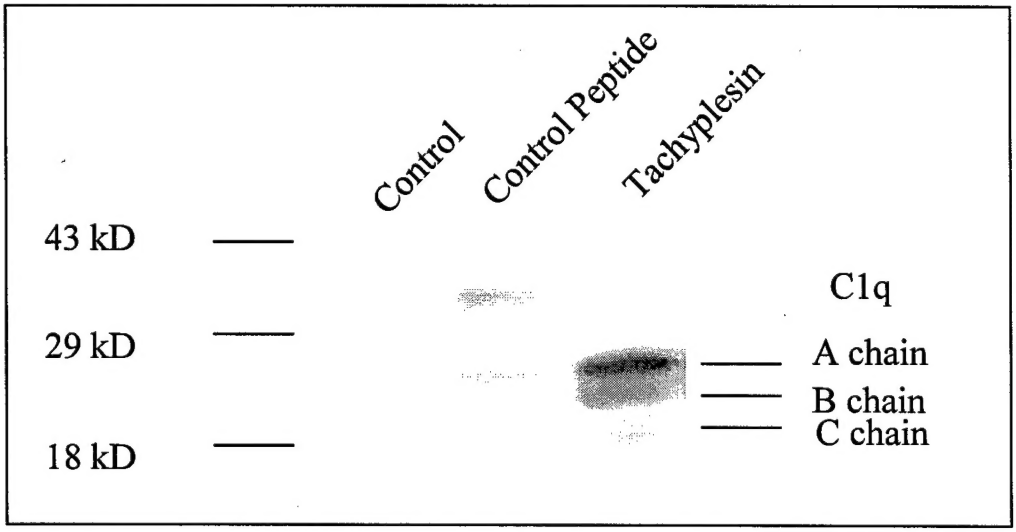

Fig. 2. Affinity-precipitation of serum C1q with tachyplesin. Normal human serum was incubated without or with biotinylated tachyplesin and control peptide, along with streptavidin-Sepharose in VSB buffer at 4 °C overnight. After washing, the beads were extracted in Laemmli sample buffer (reducing conditions) and subjected to SDS-PAGE and Western blotting with anti-C1q antibody. Three bands representing the A, B, C chains of C1q (27,550, 25,200, 23,800 Dalton, respectively) were apparent in the sample incubated with biotinylated tachyplesin but not detected in the absence of peptide or in the control peptide. The results are representative of three different experiments.

3. Activation of the Classical Complement Pathway by Tachyplesin: To determine if tachyplesin could activate the complement pathway, we used ELISAs with tachyplesin-coated microplates. Normal serum was diluted with VBS (containing Ca^{2+}) and applied to wells coated with tachyplesin or the control peptide, washed and then probed with polyclonal antibodies against C4, C3, and C5b-9. As shown in **Fig. 3 A,** the significant amounts of activated fragments of C4b, C3b, and C5b-9 complex were detected in the forms of property. Furthermore, when the same fresh serum was heat-inactivated (56 °C, 30 min) prior to the use, the activated fragments of C4b, C3b, and C5b-9 complex were detected at a level of background similar to the control peptide in the same ELISA. These data strongly demonstrates that tachyplesin is able to trigger the activation of whole classical complement cascade, since the appearance of C4b, C3b, and C5b-9 complex is the characteristics of the classical activation of complements.

To visualize the existing of activated C4b fragments, Western blotting was performed. Normal fresh human serum was mixed with the biotinylated tachyplesin or control peptide, affinity-precipitated with streptavidin-Sepharose, subjected to SDS-PAGE and finally Western blotted using antibodies to C4. **Figure 3 B** shows that the three peptide chains of C4b (α 97 kDa, β 75 kDa, and γ 33 kDa, respectively) were affinity-precipitated in the tachyplesin-treated sample, but not in those treated with the control peptide or in the absence of peptide. These results are consistent with the results obtained from ELISA, which further confirm that the tachyplesin is capable to activate the classical complement cascade, although we cannot

exclude the possibility that tachyplsein binds selectively and directly to C4b and then activate the down-stream molecules.

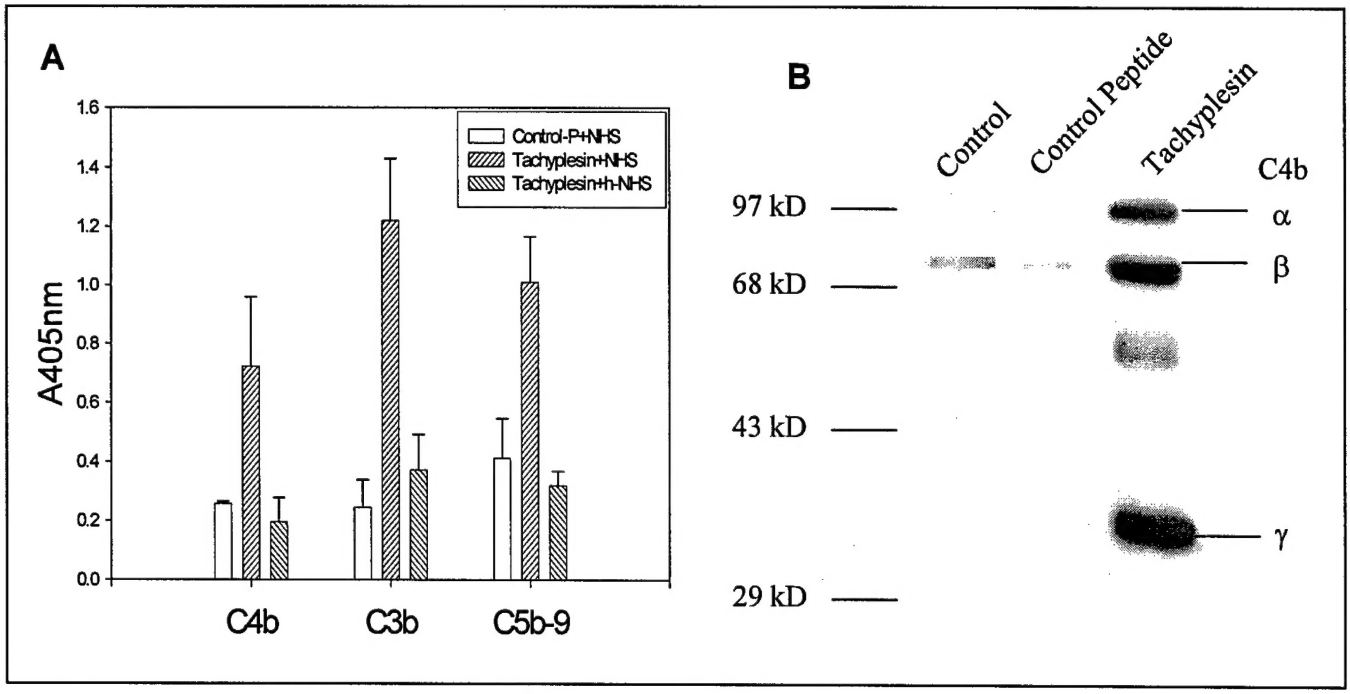

Fig. 3. Determination of tachyplesin-mediated activation of the classical complement pathway by ELISA and affinity-precipitation. A) Microtiter plates coated with either tachyplesin of the control peptide (10 μg/ml), incubated with normal human serum (NHS) without or with heat-inactivation (h-NHS). The serum was diluted with VBS (1:2 for C4b, C3b and 1:20 for C5b-9). Complement activation was then assessed using polyclonal antibodies against C4, C3 and polyclonal antibody to C5b-9. There was a significant amount of immobilized C4b, C3b and C5b-9 detected in the wells that were coated with tachyplesin and incubated with normal human serum, as compared to those coated with the control peptide or incubated with heat-inactivated serum. **B)** For affinity-precipitation and Western blotting, normal human serum was treated without peptide or biotinylated control peptide or biotinylated tachyplesin. Streptavidin-Sepharose beads were added to the mixture, washed thoroughly and processed for SDS-PAGE and Western blotting with antibodies to C4b. The bands of α, β, γ chains of C4 (*Mr* 97, 75, and 33 kDa, respectively) were apparent in the samples treated with tachyplesin, but not with the control peptide or in the absence of peptide.

4. Role of hyaluronan in the deposition of C4b and C3b on Tumor Cells: Since tachyplesin contains a hyaluronan binding motifs $[B(X)_7B]$ (19), we investigated the possibility that tachyplesin can bind to hyaluronan (both free and cell-associated). To this end, we took advantage of the fact that hyaluronan by itself does not bind to nitrocellulose but will do so in the presence proteins or peptides that bind to it (22). In the assay, tachyplsein was mixed with $[^3H]$-hyaluronan and then applied to a nitrocellulose membrane. The free $[^3H]$-hyaluronan was washed away with PBS, and the complex of $[^3H]$-hyaluronan-tachyplesin that was retained on the filter membrane was analyzed. **Figure 4 A** shows that tachyplsein binds strongly to hyaluronan, and this could be abolished by a 100-fold excess of unlabeled hyaluronan. In contrast, the control peptide showed little or no binding to $[^3H]$-hyaluronan, indicating that the binding of tachyplsein to hyaluronan was specific.

We then examined the binding of FITC-tachyplesin to tumor cells that express large amounts of hyaluronan on their surfaces (19, 23). As shown in **Fig. 4 B**, tachyplsein was distributed on the surface of the cells. This binding was significantly reduced by the addition of an excess of free hyaluronan on pre-treatment with hyaluronidase (**Fig 4 C**) as demonstrated by flow cytometer analysis. These results suggest

that hyaluronan or related molecules such as chondroitin sulfate (24) act targets for tachyplsein on the cell surface.

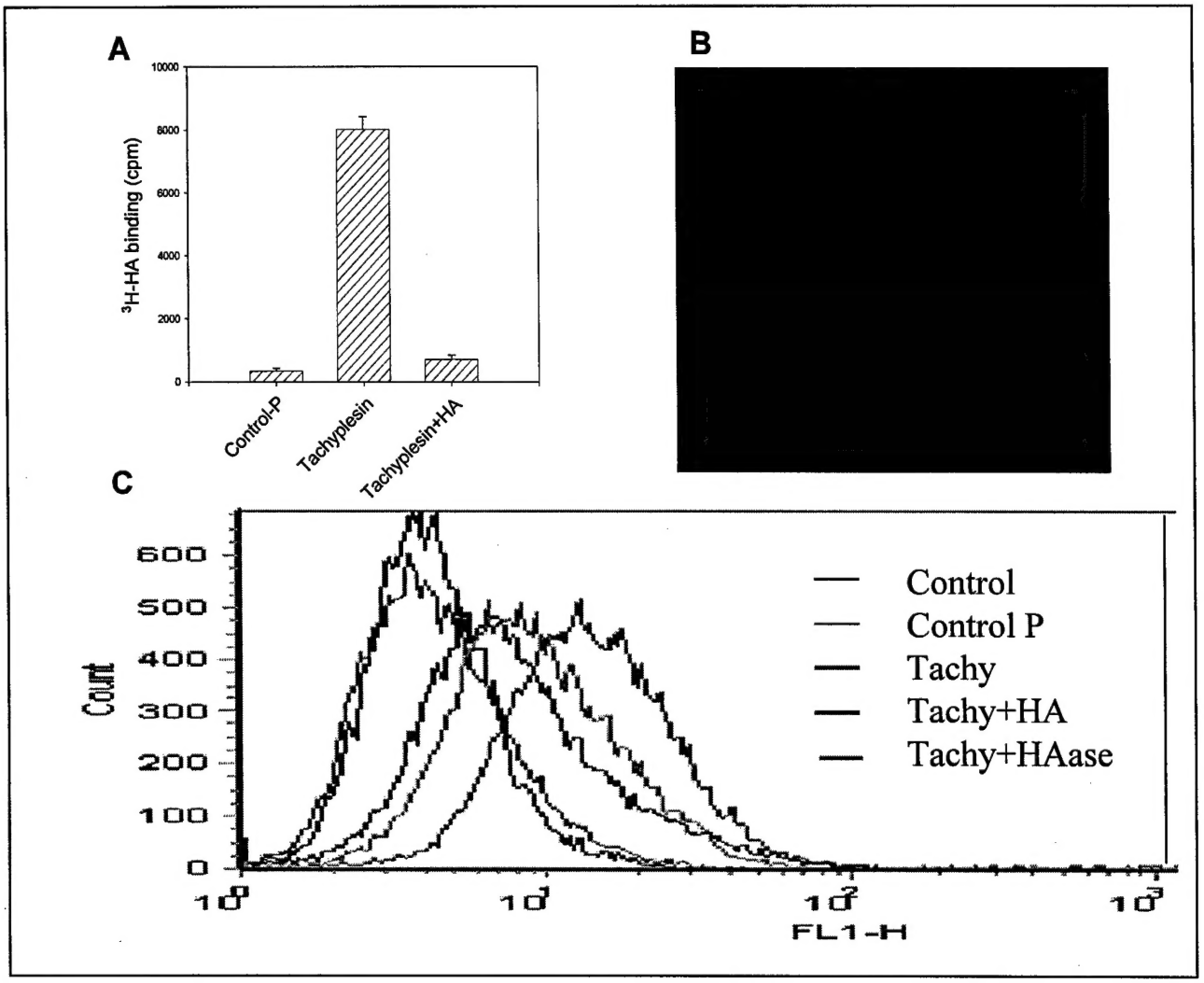

Fig. 4. Hyaluronan-mediated attachment of tachyplesin to TUMOR tumor cells. A) To determine the binding of tachyplesin to HA, tachyplesin and the control peptide were mixed with [^{3}H]-HA, applied to a sheet of nitrocellulose and extensively washed. A significant amount of [^{3}H]-HA bound to the nitrocellulose in the presence of tachyplesin but not in the control peptide or with an excess of unlabeled HA. **B)** One million tumor cells were incubated with 1 μg/ml of FITC-tachyplesin at 4 °C for 30 min, fixed with freshly prepared 4% formaldehyde and analyzed with confocal microscopy. Tachyplesin was associated with the surfaces of the cells. **C)** To determine the role of cell surface HA in the attachment of tachyplesin to tumor cells, the tumor cells were incubated with or without hyaluronidase (0.1 mg/ml) at 37 °C for 1 hour. Then, 10 μg/ml of FITC-tachyplesin was added to the cells at 4 °C for 30 min followed by fixation. Flow cytometry showed that the binding of FITC-tachyplesin to the cell surface was partially blocked by pretreatment with hyaluronidase or an excess of HA.

In the classical pathway of complement activation, 4-to 5-fold more C3b than C4b is deposited on the surfaces of target cells (17). In addition, C3 contains a thioester moiety that can form covalently bonds with nearby molecules in the transition from C3 to C3b. Thus, C3b deposition represents an index of complement activation. For this reason, we tested whether tachyplsein could induce the deposition of C3b on tumor cells. Tumor cells were incubated in a mixture of normal serum and tachyplesin, stained with

antibodies to C3 then examined by confocal microscopy. As shown in **Fig. 5 A and B**, C3b was indeed deposited on the surfaces of tumor cells.

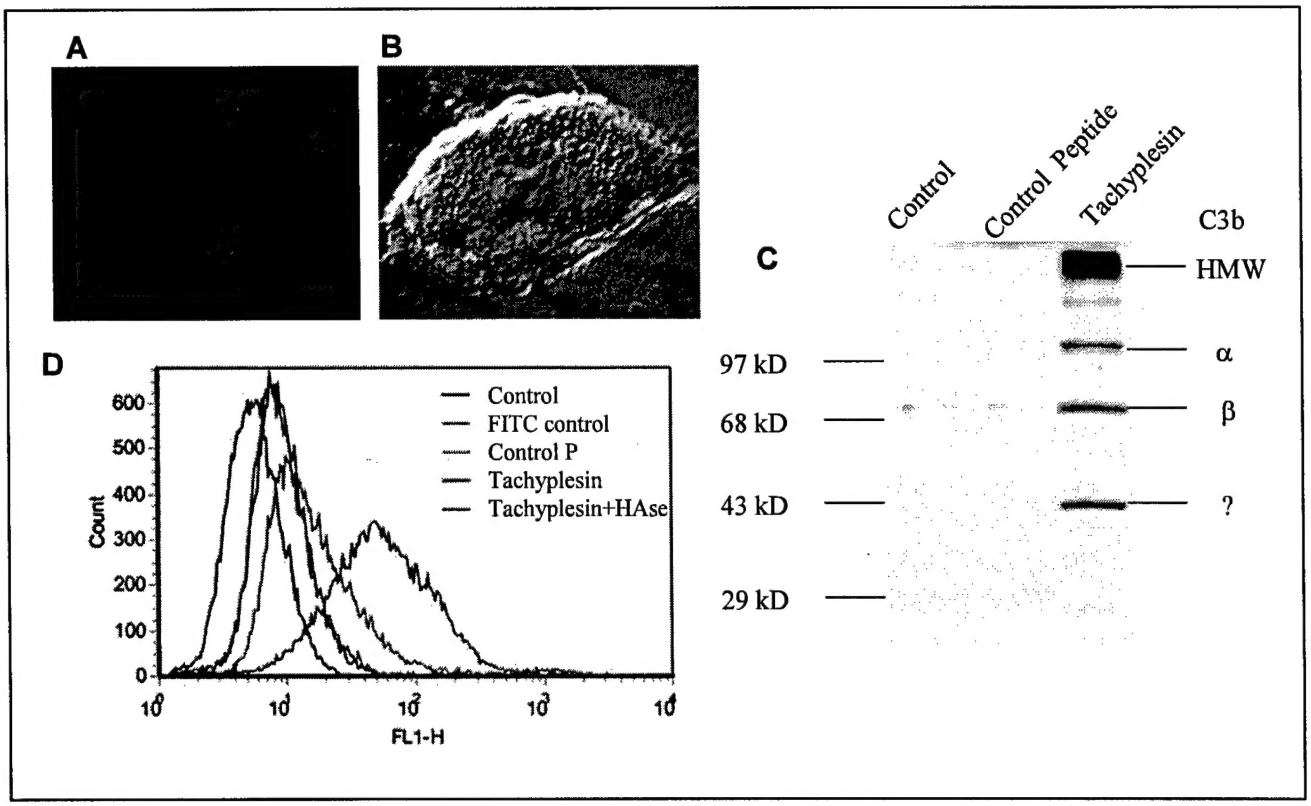

Fig. 5. Effect of tachyplesion on the induction of complement on the surfaces of tumor cells. A and B) tumor cells were incubated with tachyplesin in the presence of 10% normal human serum for 20 min, washed and then incubated with a polyclonal antibody to C3 (4 °C for 30 min) followed by a FITC-secondary antibody and analysis confocal microscopy. Both immunoflourescence alone and the merger of fluorescence and transmitted images are shown. **C)** To analyze complement activation by Western blotting, TSU cells were incubated in 10% normal human serum in the presence or absence of either tachyplesin or control peptide at 37 °C for 60 min. After washing, the cell lysates were harvested and processed for Western blotting with antibodies to C3b. In the cells treated with tachyplesin, there were two bands corresponding to α and β subunits of C3b (115 and 75 kDa), as wells as a high molecular weight band (HMW) representing C4b covalently bound to membrane constituents. These bands were reduced in cells treated without peptide or with the control peptide. **D)** To examine the role of cell surface HA in the deposition of complement, 100 μg/ml peptides were added to the cells culture in the presence of 10% normal human serum and incubated for 20 min at room temperature. The cells were then washed, incubated with a goat polyclonal antibody to human C3 (20 μg/ml) at 4 °C for 30 min followed by incubation with FITC-conjugated anti-goat IgG (1:200) at 4 °C for 30 min. The cells were finally stained with propidium iodide and analyzed with FACS. Preincubation with hyaluronidase abrogated the C3 deposition on tumor cell surfaces.

The presence of activated C3b was also demonstrated by Western blotting of tumor cells following treatment with serum and tachyplesin. **Figure 5 C** shows that in the samples treated with tachyplesin, two subunits of C3b (α 115 kDa and β 75 kDa) and possibly degraded iC3b (? band for α1). Significantly, a high molecular weight band (HMW in **Fig. 5 C**) was found with Western blotting under reducing conditions, indicating a covalent linkage to large membrane constituents. Scans of these Western blots revealed that the majority of deposited C3b (70% to 80%) was present in this high molecular weight form. This is consistent

with the fact that in the transition from C3 to C3b, a thioester moiety can form covalently bonds with nearby molecules. FACS analysis of cells treated with tachyplsein and serum (**Fig. 5 D**) showed that there was a significant increase in FITC-tagged antibody to C3, indicating that C3b deposition on the tumor cells. This did not occur with cells treated with the control peptide. These results are consistent with those from confocal microscopy and Western blotting.

Since tachyplsein can bind to hyaluronan, we investigated the possibility that membrane-bound hyaluronan plays a role the binding of tachyplesin-mediated activation of complement on the surface of tumor cells. The suspensions of tumor cells ere pretreated with hyaluronidase before the addition of tachyplsein and human serum, and the presence of C3b was detected immuno-staining followed by FACS analysis. As shown in **Figure 5D**, hyaluronidase pre-treatment markedly reduced the intensity of fluorescence, indicating a reduction in C3b deposition. Thus, hyaluronan or related glycosaminoglycans do appear to play a key role in the activation of complement on the cell surface by tachyplesin.

5. Effect of tachyplsein on Tumor Cells: The fact that tachyplsein can trigger the deposition of complement on the surfaces of tumor cells via classical pathway, suggesting that it might kill cells by disrupting the integrity of the plasma membrane. To test this possibility, we examined the permeability of cell membrane with macromolecule FITC-Dextran, which is excluded by the membranes of viable, healthy cells but can pass through the damaged plasma membrane of unhealthy cells (2). **Figure 6A** showed that when cells were treated with tachyplsein and human serum, the fluorescence spectrum was shifted, indicating that more FITC-Dextran had passed through the plasma membrane and entered the cytoplasm. Thus, it appears that treatment with tachyplsein disrupted the cell membrane and increased its permeability.

Finally we examined the effect of tachyplsein and normal human serum on cell growth. As indicated in **Figure 6 B**, tumor cells treated with tachyplsein showed a marked inhibition of proliferation in the presence of complete human serum. However, treatment of the cells with hyaluronidase significantly reversed this effect, again suggesting that cell-surface hyaluronan plays a critical role in tachyplesin-induced inhibition of tumor cell growth. Significantly, heat-inactivated serum also attenuated the effects of tachyplesin, but to a lesser extent than hyaluronidase treatment. These results imply that tachyplsein may have multiple effects on the cells leading to both growth arrest and death.

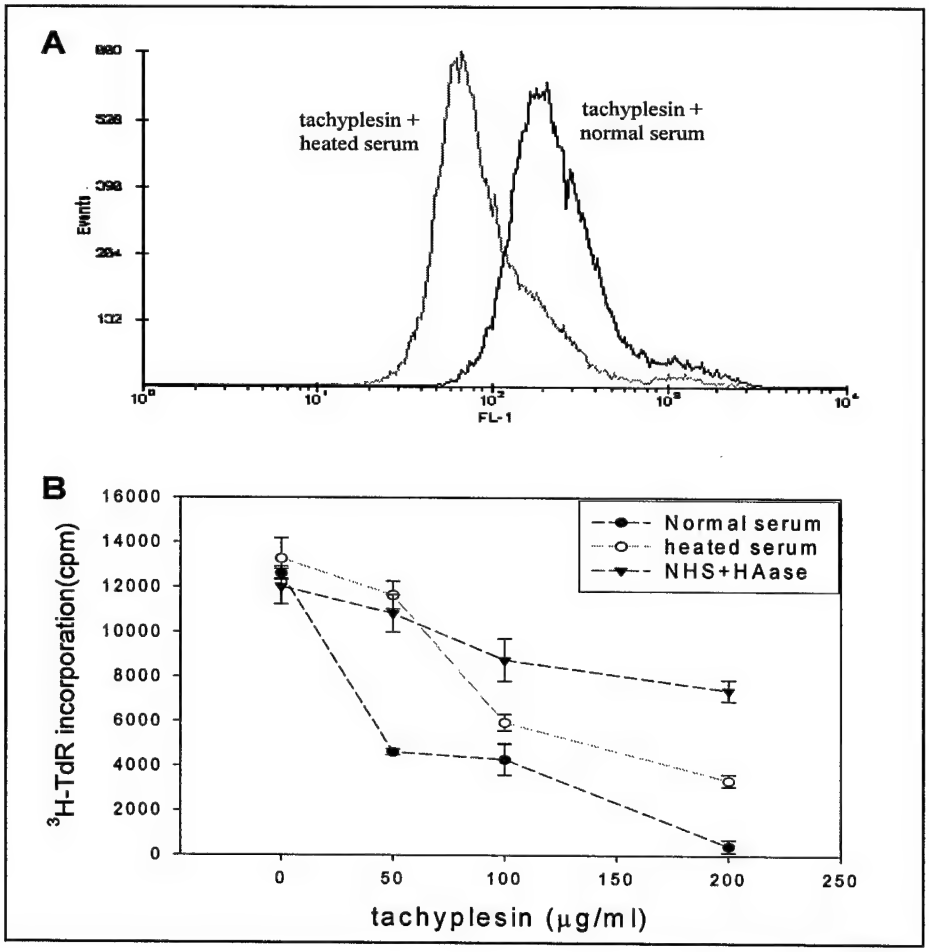

Fig. 6. Biological Effect of hyaluronidase on tachyplesin-mediated cytotoxicity of tumor cells. A) To determine whether tachyplesin damages the plasma membrane, TSU cells were incubated with tachyplesin plus or heat-inactivated human serum or normal human serum (NHS) overnight. After harvesting, the cells were incubated with 5 μg/ml of FITC-Dextran (*Mr* 40,000) and subject to flow cytometry. The amount of FITC-Dextran taken up by the cells was significantly higher in those treated with tachyplesin than in the control cells, suggesting that tachyplesin disrupts the integrity of the plasma membrane. **B)** Tumor cells were treated with hyaluronidase (0.1 mg/ml) at 37 °C for 1 hour. Then, tachyplesin and 2% normal human serum or heat-inactivated serum were added and incubated overnight followed by a [^{3}H]-thymidine incorporation assay. The cytotoxic effects of tachyplesin in present of normal serum were reduced by either treatment with hyaluronidase or heat-inactivated serum.

Taken together, these observations suggest that HABP / tachyplesin binds to both hyaluronan on the cell surface and C1q in the serum and activates the classical complement cascade, which damages the integrity of the membranes of the tumor cells resulting in their death.

Key Research Accomplishments

In past year, we have accomplished the follow tasks: **1)** isolate and characterize the HABP/tachyplesin-binding phages; **2)** define the binding of the C1q to tachyplesin; **3)** elucidate the activation of the classical complement pathway by HABP/tachyplesin; **4)** define the role of hyaluronan in the deposition of C4b and C3b on tumor cells; and **5)** further examine the effect of HABP/tachyplesin on tumor cells.

Conclusions

1. HABP/tachyplesin binds to C1q on cell surface.
2. HABP/tachyplesin appears to activate the classical complement cascade, since it triggered several down-stream events including the cleavage and deposition of C4 and C3 and the formation of C5b-9.
3. The HABP/tachyplesin binding to C1q and activation of classical complement cascade can be blocked if the tumor cells are treated with hyaluronidase or a large excess of hyaluronan, indicating that hyaluronan or related glycosaminoglycans were involved in this process.
4. Treatment of cells with HABP/tachyplesin and serum results in an increase in membrane permeability as indicated by the ability of FITC-dextran to enter the cytoplasm.
5. The combination of HABP/tachyplesin and human serum can markedly inhibit the proliferation of tumor cells and this effect is attenuate if the serum is heat-inactivated or if hyaluronidase is added.
6. This represents a new anti-tumor mechanism.

Reportable outcomes

(Due to or partially due to this support)

Papers

1. Ninfei Liu, Xue-Ming Xu, Jinguo Chen, Luping Wang, Shanmin Yang, Charles B. Underhill, and Lurong Zhang: A Hyaluronan-Binding Peptide Can Inhibit Tumor Growth by Interacting with Bcl-2. International Journal of Cancer 2004; 109 : 49-57

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4. Jinguo Chen, Xue-Ming Xu, Charles B. Underhill, Shanming Yang, Luping Wang, Yixin Chen, Shuigen Hong, Karen Creswell, Lurong Zhang: Tachyplesin Activates the Classical Complement Pathway to Kill Tumor Cells (submitted to Cancer Res.)

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1. Weimin Liu, Ivan Ding, Keqiang Chen, John Olschowka, Lurong Zhang and Paul Okunieff:Elevation of IL-1β and matrix metalloproteinases (MMPs) in radiation induced mouse dermatitis and fibrosis. Proc. Annu. Meet. Am. Assoc. Cancer Res. 2004; 3119

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6. Jinguo Chen, Xue-Ming Xu, Charles B. Underhill, Shanming Yang, Karen Creswell and Lurong Zhang: Tachyplesin can kill tumor cells by activation of the classical complement pathway. Proc. Annu. Meet. Am. Assoc. Cancer Res. 2004; 4375

7. Shanmin Yang, Jinguo Chen, Charles B. Underhill, Sunghee Kim, Luping Wang, Xu-Ming Xu and Lurong Zhang: Effect of Triptolide on the Telomerase Activity in Human Cancer Cells. Proc. Annu. Meet. Am. Assoc. Cancer Res. 2004,993

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